Microarray analysis of *Streptococcus pneumoniae* gene expression changes to human lung epithelial cells

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Abstract: *Streptococcus pneumoniae* infection starts from the respiratory tract where interaction with host epithelial cells occurs. To gain more insights on pneumococcal pathogenesis, an oligonucleotide (oligo)-based microarray was used to investigate gene expression changes of one serotype 3 encapsulated pathogenic *S. pneumoniae* strain 82 and one unencapsulated avirulent *S. pneumoniae* strain R6 upon exposure to human lung epithelial cells (A549) for 1 and 3 h, respectively. We observed that genes associated with many functional categories were differentially regulated in strain 82, such as genes in pathogenesis, cell envelope, transcription, translation, transport, metabolism, and unknown functions. In contrast, few genes were changed in strain R6 except for genes in ribonucleotide biosynthesis and unknown functions. Quantitative real-time PCR analysis confirmed the microarray results for most of the genes tested. To further characterize functions of the selected genes, knockout mutants were constructed in strain R6. We demonstrated that 2 genetic loci, SP_2170 (*AdcB*, zinc ABC transporter) and SP_0157 (hypothetical protein), were involved in adherence to A549 cells. These data suggest that divergent gene expression changes occur in *S. pneumoniae* pathogenic and avirulent strains during interaction with human lung epithelial cells. Some of those genes are involved in pneumococcal pathogenesis.

Key words: Streptococcus pneumoniae, microarray, epithelial cells, adherence.

Résumé : L'infection à *Streptococcus pneumoniae* commence dans les voies respiratoires suite à son interaction avec les cellules épithéliales de l'hôte. Afin de mieux comprendre la pathogenèse des pneumocoques, un essai sur des micropuces d'oligonucléotides a été utilisé pour examiner les changements d'expression génique d'une souche pathogène de sérotype 3 encapsulée, la *S. pneumoniae* souche 82, ainsi que d'une souche non-encapsulée, non virutente, la *S. pneumoniae* souche R6, en réponse à des expositions de 1 ou 3 h respectivement des cellules épithéliales de poumon humain A549. Nous avons observé que des gènes associés à différentes catégories fonctionnelles sont réglés de façon différentielle chez la souche 82, tels des gènes associés à la pathogenèse, à l'enveloppe cellulaire, à la transcription, à la traduction, au transport, au métabolisme, ainsi que des gènes de fonction inconnue. Au contraire, peu de gènes étaient affectés dans la souche R6, à l'exception de gènes impliqués dans la biosynthèse de ribonucléotides et de gènes de fonction inconnue. Une analyse par PCR quantitative en temps réel (qRT-PCR) a confirmé les résultats obtenus sur micropuces pour la plupart des gènes testés. Afin de caractériser davantage les fonctions de certains gènes choisis, des mutants knock-out ont été construits à partir de la souche R6. Nous avons démontré que deux loci génétiques, SP_2170 (transporteur ABC de zinc *AdcB*) et SP_0157 (protéine hypothétique), sont impliqués dans l'adhérence aux cellules A549. Ces résultats suggèrent que des changements d'expression divergents se produisent dans les souches de *S. pneumoniae* pathogènes et non virulentes lors de leur interaction avec les cellules épithéliales du poumon. Quelques uns de ces gènes sont impliqués dans la pathogenèse des pneumocoques.

Mots-clés : Steptococcus pneumoniae, micropuce, cellules épithéliales, adhérence.

[Traduit par la Rédaction]

Introduction

Streptococcus pneumoniae is a major bacterial pathogen in humans, causing respiratory tract diseases, such as pneumonia, bronchitis, otitis media, and sinusitis, and invasive infections, such as bacteremia, sepsis, meningitis, peritonitis, and arthritis. Despite the availability of antibiotics and vaccines, approximately 1.1 million deaths worldwide are annually attributed to pneumococcal infections (Klein 1999).

Streptococcus pneumoniae infection starts from the colonization of the human upper respiratory tract, where interaction with mucosal epithelial cells occurs. Therefore, the interaction of *S. pneumoniae* and host respiratory tract epithelial cells is essential for infection. Many bacterial factors that contribute to the adherence and (or) invasion of host respiratory tract epithelial cells have been characterized in

Received 17 September 2007. Revision received 7 November 2007. Accepted 10 December 2007. Published on the NRC Research Press Web site at cjm.nrc.ca on 23 February 2008.

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 doi:10.1139/W07-133

S. pneumoniae, such as pneumococcal surface adhesin A (PsaA), pneumococcal adherence and virulence factor A (PavA), IgA protease, autolysin (LytA), neuraminidase (NanA/NanB), pneumolysin (Ply), hyaluronate lyase (Hyl), and choline-binding proteins CbpA, LytA, PspA, and PspC (recently reviewed by Bergmann and Hammerschmidt (2006); Hammerschmidt (2006); Mitchell (2006)). However, it is becoming obvious that multiple factors are involved in the initial process of colonization of the host respiratory tract epithelial cells because knockouts of individual proteins could reduce but not abolish such effects (Hammerschmidt et al. 2005).

To know more about pneumococcal genes that are associated with pathogen–host interactions and to identify novel gene targets that are associated with adherence, we utilized a microarray approach to study pneumococcal gene expression changes during pathogen–host interactions. In this report, we developed a *S. pneumoniae* oligonucleotide (oligo)-based microarray. By using these microarrays, we investigated pneumococcal gene expression changes in 2 strains upon exposure to human lung epithelial cells. Subsequently, we characterized 3 differentially regulated genes by mutagenesis and adherence analyses. Two genetic loci were demonstrated to be involved in adherence to host epithelial cells.

Materials and methods

Bacterial strains, epithelial cell, and culture conditions

Strain 820047, renamed 82 in this study, was kindly provided by Dr. Donald E. Low of Mount Sinai Hospital in Toronto, Ontario, Canada. It is an encapsulated serotype 3 pneumococcal strain isolated from a patient with chronic obstructive pulmonary disease. *Streptococcus pneumoniae* R6, obtained from American Type Culture Collection (ATCC), is an unencapsulated and avirulent strain derived from encapsulated serotype 2 pathogenic strain D39. *Streptococcus pneumoniae* strains were cultured in Todd–Hewitt culture medium (Oxoid) supplemented with 0.5% (*m*/*v*) yeast extract (THY) at 37 °C in a 5% (*v*/*v*) CO_2 incubator. Human lung epithelial cell line A549 (ATCC No. CCL-185, VIDO collection) was cultured in MEM complete medium consisting of Minimum Essential Medium (MEM) (Gibco), 10% (*v*/*v*) fetal bovine serum, 10 mmol·L^{-1} of non-essential amino acid solution (Gibco), 10 mmol·L^{-1} of HEPES (Sigma) and 50 μg·mL^{-1} of gentamicin (Gibco). When used for bacterial infection, host cells were prewashed once with MEM and maintained in antibiotic-free MEM complete medium with 1% (*v*/*v*) fetal bovine serum for 3 h.

Pneumococcal DNA isolation and transformation

Streptococcus pneumoniae genomic DNA was isolated with a DNeasy Tissue Kit (Qiagen). Transformation of *S. pneumoniae* was performed according to a previously described method (Johnston et al. 2006) with minor modifications. Briefly, the recipient pneumococcal strain was grown in THY medium supplemented with 0.5% (*m*/*v*) glycine to logarithmic phase at an OD_{600} absorbance of 0.3. Bacteria were then diluted 10-fold in CTM competence medium (THY medium supplemented with 0.2% (*m*/*v*) BSA, 0.2% (*m*/*v*) glucose, and 0.02% (*m*/*v*) $CaCl_2$), and the competence was induced with 500 ng·mL^{-1} of competence-stimulating peptide CSP1 or CSP2 (CSP-1: EMRLSKFFRDFILQRKK; CSP-2: EMRISRIILDFLFLRKK; synthesized at VIDO) for 14 min prior to the addition of purified PCR fragments.

Mutagenesis in *S. pneumoniae*

A modified 3-step PCR mutagenesis method (Lau et al. 2002; Song et al. 2005) was used in this study. (1) Amplification of an erythromycin (Erm) resistance cassette, kindly provided by Dr. Donald A. Morrison of University Illinois at Chicago, with the primer pairs DAM212 and DAM213 (Table 1). (2) Amplification of the left and right flanking regions of the target genes, at a length of between 800 and 1200 bp, with primer pairs L-F/L-R-Erm and R-F-Erm/R-R, respectively. Primers L-R-Erm and R-F-Erm each contain 27 or 28 nucleotides complementary to the 5′-end and 3′-end sequences of the Erm resistance cassette. (3) Fusion of the amplified left and right flanking regions to the amplified Erm cassette by running another PCR reaction with primer pairs L-F and R-R. PCR primers were designed from *S. pneumoniae* TIGR4 genome sequence (TIGR) and synthesized by Invitrogen (Table 1). The PCR amplification was performed on a Thermal Cycler (Applied Biosystems) using r*Taq* DNA Polymerase (GE Healthcare) or *Taq* Advantage 2 Polymerase Mix (Clontech). The PCR products were purified with agarose electrophoresis using QIAquick Gel Extraction Kit (Qiagen). Mutants were selected from colonies resistant to Erm (1 μg·mL^{-1}), and the gene replacement was further examined by PCR for the insertion of the Erm cassette and for the changes in size in the mutated regions.

Bacterial adherence assay

Bacterial adherence to A549 cells was characterized in 6-well cell culture plates (Corning) using a previously described method (Song et al. 2004) with minor modifications. Following incubation with bacteria, host cells were washed 3 times with 0.1 mol·L^{-1} phosphate-buffered saline buffer (PBS) and lysed with a PBS buffer supplemented with 1% (*m*/*v*) saponin (Sigma) and 0.025% (*m*/*v*) trypsin. Bacterial colony-forming unit (CFU) was determined on THY agar plates. All the assays were performed separately for at least 3 times, and statistical significance was calculated with PRISM (GraphPad) using a two-tailed unpaired *t*-test.

Collection of bacterial samples for transcriptional analyses

For microarray analysis, logarithmic-phase bacterial cultures were diluted in antibiotic-free MEM complete medium with 1% (*v*/*v*) fetal bovine serum. For better contact with host cells, a small volume (1.5 mL) of bacterial solution was incubated with A549 cells grown in a T75 cell culture flask (Corning) at a multiplicity of infection range of 10:1 – 50:1. After incubation for 1 or 3 h, the bacterial solution was removed and 5 mL of host cell lysis buffer consisting of RLT lysis buffer (Qiagen), 1% β-mercapthoethanol, 1% (*v*/*v*) phenol, and 10% (*v*/*v*) ethanol was added immediately. The RLT lysis buffer was used to remove host cell contamination (Di Cello et al. 2005), while the phenol and ethanol were used to stabilize bacterial RNA (Gaynor et al. 2004). Following incubation at room temperature for 10 min, host

Table 1. Synthesized oligonucleotide primers used for mutant construction and characterization.

Primer	Primer sequence 5′ to 3′[a]	Description
DAM212[b]	CCGGGCCCAAAATTTGTTTGAT	Forward primer, used to amplify Erm resistance cassette
DAM213[b]	AGTCGGCAGCGACTCATAGAAT	Reverse primer, used to amplify Erm resistance cassette
SP_0157_L-F	CTTTGAACGCTGGAAGCTGAATGC	Forward primer, used to amplify left flanking region of SP_0157 for the construction of ΔSP_0157 mutant
SP_0157_L-R-Erm	TACAAATCAAACAAATTTTGGGCCCGGGCACGAAGGGCTTCTTTACTAGGC	Reverse primer, carrying a 27 bp sequence complementary to the 5′ end of the Erm resistance cassette, used to amplify left flanking region of SP_0157 for the construction of ΔSP_0157 mutant
SP_0157_R-F-Erm	AAATAATTCTATGAGTCGCTGCCGACTATGGCGTGGATAACGGAGATGTTG	Forward primer, carrying a 27 bp sequence complementary to the 3′ end of the Erm resistance cassette, used to amplify right flanking region of SP_0157 for the construction of ΔSP_0157 mutant
SP_0157_R-R	GTATGCCTCTGTTCTTGGCGAATGG	Reverse primer, used to amplify right flanking region of SP_0157 for the construction of ΔSP_0157 mutant
SP_0416_L-F	TTACCCGCAACATAGTCGCGGTAG	Forward primer, used to amplify left flanking region of SP_0416 for the construction of ΔSP_0416 mutant
SP_0416_L-R-Erm	TACAAATCAAACAAATTTTGGGCCCGGTCTGCCGTTAAAGCCTCTCCTGTC	Reverse primer, carrying a 27 bp sequence complementary to the 5″ end of the Erm resistance cassette, used to amplify left flanking region of SP_0416 for the construction of ΔSP_0416 mutant
SP_0416_R-F-Erm	AAATAATTCTATGAGTCGCTGCCGACTGCGAGGAAGAAATTGCTGTCATGG	Forward primer, carrying a 27 bp sequence complementary to the 3′ end of the Erm resistance cassette, used to amplify right flanking region of SP_0416 for the construction of ΔSP_0416 mutant
SP_0416_R-R	CACCGAAGCCTGATAGAAGAACAGTC	Reverse primer, used to amplify right flanking region of SP_0416 for the construction of ΔSP_0416 mutant
SP_2170_L-F	TCCTTGACAGCTGCATCCTCGAA	Forward primer, used to amplify left flanking region of SP_2170 for the construction of ΔSP_2170 mutant
SP_2170_L-R-Erm	GAAATAATTCTATGAGTCGCTGCCGACTATCGGTTATGCTGCTTGCCAGTG	Reverse primer, carrying a 28 bp sequence complementary to the 3′ end of the Erm resistance cassette, used to amplify left flanking region of SP_2170 for the construction of ΔSP_2170 mutant
SP_2170_R-F-Erm	ATACAAATCAAACAAATTTTGGGCCCGGACTCTGACGACGCAAGATGAGG	Forward primer, carrying a 28 bp sequence complementary to the 5′ end of the Erm resistance cassette, used to amplify right flanking region of SP_2170 for the construction of ΔSP_2170 mutant
SP_2170_R-R	ATGTCAGTCAGGCGGCAGTTAC	Reverse primer, used to amplify right flanking region of SP_2170 for the construction of ΔSP_2170 mutant

[a]The nucleotide sequences complementary to the Erm resistance cassette are underlined.
[b]Sequences were obtained from Dr. Donald A. Morrison.

cells were lysed and bacteria were collected for RNA isolation by centrifugation at 20 000*g* for 10 min. The medium control bacterial samples were prepared by incubation of bacterial cultures under the same condition without host cells, followed by treatment with RNAlater (Ambion).

For the analysis of polar effects of the mutant strains with quantitative real-time PCR (qRT-PCR), both wild-type and mutant strains were cultured in THY broth to logarithmic phase and collected for RNA isolation following treatment with RNAlater.

RNA isolation and purification

Total RNA from *S. pneumoniae* strains incubated with A549 cells was isolated with RiboPure™-Bacteria Kit (Ambion). Owing to a low yield of RNA when using the same kit for the RNAlater treated samples (data not shown), total RNA from other samples was isolated with an RNeasy MiniKit (Qiagen) supplemented with a pretreatment of 10 mg·mL^{-1} lysozyme and 250 units·mL^{-1} mutanolysin (Sigma) for 10 min at 37 °C. Genomic DNA contamination was removed by treating with RNase-free of DNase I (Ambion). The integrity, purity, and concentration of RNA samples were determined with capillary electrophoresis on a Bioanalyzer (Agilent Technologies). From each flask of cells incubated with bacteria, approximately 1.5–4 μg of total bacterial RNA at a purity of 70%–95% could be generated.

cDNA synthesis and labelling

For microarray studies, cDNA samples were synthesized and labelled with a LabelStar Array Kit (Qiagen) using random hexamer primers (Applied Biosystems). For each microarray slide analysis, 1 μg of total RNA was required. RNA samples from bacteria incubated with host cells and culture media were labelled with biotin–dUTP and fluorescein–dUTP (Enzo), respectively. After purification on the Cleanup columns (Qiagen), labelled cDNA samples were mixed and hybridized to the same microarray slide. Labelling swap, i.e., switch of cDNA labelling, was not applied in this study. For qRT-PCR analysis, cDNA samples were synthesized with a SuperScript III Reverse Transcriptase (Invitrogen) using the same random primers.

Development of *S. pneumoniae* oligo microarray

The 2030 *S. pneumoniae* microarray oligo probes were designed from the *S. pneumoniae* TIGR4 genome (TIGR) using the ROSO software (http://pbil.univ-lyon1.fr/roso/) (Reymond et al. 2004). They include 1995 probes selected from all ORFs longer than 150 bp in the TIGR4 genome, 28 unique genes from the *S. pneumoniae* R6 genome, 2 unique antibiotic resistance genes from other pneumococcal strains, and 5 eukaryotic cell control genes. The oligo probes were selected from the DNA sense strand and had a length of 50 nucleotides and a T_m of 80 ± 5 °C. To avoid cross-hybridization with eukaryotic cell RNA, all the oligo probe sequences were compared against the human genome sequences. Except for a few transposase genes, the shared homology with any human transcripts and selected oligos was less than 70%. The microarray oligos were synthesized by Operon Biotechnologies and spotted onto the GAP II glass slides (Corning) in triplicates by the University of Minnesota Microarray Facility.

Microarray analysis

Microarray hybridization and detection analysis was performed with a Resonance Light Scattering (RLS)-based system by using a Two-Colour Nucleic Acid Microarray Toolkit (Invitrogen). The scattered light signals of RLS particles Ag and Au on the microarrays were scanned with a GSD-501 RLS imager using an ICS-501 (version 2.3) Image Capture System (Invitrogen). The gridding of spots was performed with ArrayVision 8.3 (Imaging Research). The estimated background value for each spot was the median of measurements made at the 4 corners of each spot, and the background was subtracted from corresponding spot intensity. The microarray data were flagged and normalized with Limma Loess (applied to each subgrid) and statistically analyzed with Student's *t* test using ArrayPipe software (http://www.pathogenomics.ca/arraypipe) (Hokamp et al. 2004). The genes with a fold change of ≥2.0 and a statistical significance of $P \leq 0.05$ were classified as significantly changed. In this study, the microarray experiments were repeated 5 times with separate isolated samples for each time point, except for the 4 repeats for the strain 82 samples incubated with A549 for 1 h. To comply with the MIAME (Minimum Information About a Microarray Experiment) requirement, microarray data have been deposited in the ArrayExpress microarray database (http://www.ebi.ac.uk/arrayexpress) under accession No. E-TABM-187.

Quantitative real-time PCR (qRT-PCR) analysis

The oligo primers used for qRT-PCR studies (Table 2, synthesized by Invitrogen) were designed from either *S. pneumoniae* TIGR4 or R6 genome sequences by using Clone Manager Suite 7 (Scientific and Educational Software). The qRT-PCR reaction was performed on 96-well plates with an iCycler™ iQ real-time PCR detection system (Bio-Rad). In a 15 μL reaction volume, 9 μL of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) was mixed with 1 μL (10 μmol·L^{-1}) of each gene-specific sense and anti-sense primers. The cDNA derived from 20–40 ng of total RNA was used for each reaction. The qRT-PCR cycling program included 45 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s after holding for 2 min at 50 and 95 °C, respectively. A melting curve was obtained by adding the following steps after PCR amplifications: 95 °C for 1 min, 55 °C for 1 min, and 40 cycles increasing the setpoint temperature by 1 °C. For each gene, duplicate or triplicate qRT-PCR reactions were performed on the RNA samples isolated from at least 3 separate experiments for each time point or from strains grown in THY medium. The relative fold change of each gene was calculated (Livak and Schmittgen 2001) by using *gyrA* as an internal control gene. Genes with a fold change of ≥2.0 from all the individual samples and that were regulated in the same direction (up- or down-regulation) were regarded as significantly changed.

Results

Divergent transcriptional profiles of pneumococcal strains

At the beginning of this project, we examined pneumococcal RNA yield from a few clinical isolates in our collec-

Table 2. Synthesized oligonucleotide primers used for qRT-PCR analysis.

Gene name	*Streptococcus pneumoniae* genome acc. No.[a]	Sense primer 5′ to 3′	Anti-sense primer 5′ to 3′	Amplified product (bp)
	SP_0158[b]	TTTGGTGTGGTTGGGTCAGGAAATCG	AAGTCAGCCAATACAGGGAAGCCAAAGC	101
	SP_0159[b]	ACCATTCGCCAAGAACAGAGGCATAC	CAGCTATCCTAGCCGTCTTCTTTGTG	135
bgaA	SP_0648	CAAGCCAGCCGTGAACGCTATAAGG	GAGTGGGCAGTCAGGGTGAATTTCC	128
gyrA	SP_1219	GTGCTGCCGCTCAACGTTATACCGAGG	AAACGCGCTGGCAAGACCAAGGGTTCC	142
pck	SP_1269	AATGTGGGATTTGGCTGCCCTCTT	GAGAAACCGGTGTTTGGTCACTCTCA	101
hprK	SP_1413	GCCGTGCAGCTACCAGTCGTTTATC	TCTCCCTGAATCAAGACGCCCATCC	127
ply	SP_1923	ATCCTGGAGCACTTCTCGTAGTGGATG	GGGTCTTCCACTTGGAGAAAGCTATCG	142
adcA	SP_2169[b]	GCTGACAAGCTGTCGCGGATGTG	AGGTGGCGAGGAAGAAGAGGGAG	132
purF	spr0047	ATCCACGGTGTCAATGTCCATACG	GTAAGCCTGCTTCTTCCGCAAATC	145
purK	spr0054	GGCACCCTACAAGGTCGTGACTTC	GTAGCCACCAGTCGCAGTCTTGAG	91
carA	spr1154	TATCCAGCTCCAGGAGTTGGTTTG	GGAGAATTTCTTCAGCCGTTGTCG	136
groES	spr1723	TGTGGCTACTGGACAAGGTGTTCG	ACCTGCGTGGGCTTCAACTAAGAC	97
purR	spr1793	AGGCAGCCGTGTCTTGATTGTGG	TTGTCCGCAAAGACCGCTACACC	120

[a]All primers were designed from genome sequences of either *S. pneumoniae* TIGR4 (SP_) or *S. pneumoniae* R6 (spr).
[b]The sequence that is identical to the homolog genes in *S. pneumoniae* R6 genome (spr).

Fig. 1. Gene expression changes of strain 82 (A) and strain R6 (B) identified in microarray and qRT-PCR analyses. Standard deviation is indicated by the vertical bars. Scales on the *y*-axis are not continuous because of the large fold change for some genes.

tions under defined experimental conditions. The serotype 3 strain 82 was one of the strains generating relatively higher yield (data not shown). Considering its importance in the clinic (see Discussion), a serotype 4-derived strain has been studied with microarray analysis (Orihuela et al. 2004), we selected strain 82 in this study. To investigate genes that might be associated with pathogenesis, transcriptional profiles of an avirulent strain R6 was also analyzed. In this study, by comparing strains incubated with host cells and media, we observed genes associated with many functional categories that were differentially regulated in strain 82, such as genes in pathogenesis and toxins, cell envelope, transcription, regulation, translation, transport, metabolism, and unknown functions. In contrast, most of those categories of genes, except for functional unknowns, were unchanged in strain R6. However, genes in ribonucleotide biosynthesis were down-regulated in strain R6 (Table 3). Furthermore, we also observed a time-dependent gene expression change pattern. One typical example was that only 2 genes were identified at the 3 h incubation time period in strain R6.

Validation of gene expression changes

To confirm the microarray results, we used qRT-PCR analysis to examine the gene expression changes of 9 genes (8 changed and 1 unchanged in microarray studies) from the same samples that were used for microarray analysis. Except for the *hprK* (SP_1413) gene, which was changed in microarray ($F > -2.0$, $P < 0.05$) but not in qRT-PCR ($F < -2.0$),

Table 3. Differentially regulated genes of *Streptococcus pneumoniae* identified in microarray studies.

Function and gene name	Protein	TIGR4 acc. No.	Strain 82		Strain R6	
			1 h	3 h	1 h	3 h
Pathogenesis and toxins						
blpK	Bacteriocin BlpK	SP_0533	–2.2			
bgaA	β-Galactosidase	SP_0648		2.5		
ply	Pneumolysin	SP_1923		–4.0		
Cell envelope						
licB	LicB protein	SP_1268	2.6	2.6		
pck	Choline kinase	SP_1269	3.3			
	Alcohol dehydrogenase, zinc-containing	SP_1270	3.3			
	2-*C*-Methyl-D-erythritol 4-phosphate cytidylyltransferase	SP_1271	3.5			
Transcription						
rnc	Ribonuclease III	SP_1248	3.3			
	Transcriptional regulator	SP_1809	–2.4			
MarR family	Transcriptional regulator	SP_0416	–2.4	–3.7		
Regulatory functions						
hprK	Hpr(Ser) kinase-phosphatase	SP_1413	–2.3			
phoU	Phosphate transport system regulatory protein	SP_1395	–2.2		–2.2	
Stress and protein fate related						
groEL	Chaperonin, 60 kDa	SP_1906	–3.1			
groES	Chaperonin, 10 kDa	SP_1907	–5.9	–2.2	–2.9	
	General stress protein 24	SP_1804	–3.5			
	Serine protease	SP_2239			–2.0	
	ATP-dependent Clp protease, ATP-binding subunit	SP_0338				2.9
Translation						
rplT	Ribosomal protein L20	SP_0961	4.5			
rpmG-2	Ribosomal protein L33	SP_2009	–3.7		–3.5	
rplB	Ribosomal protein L2	SP_0212		–3.3		
rpsS	Ribosomal protein S19	SP_0213		–3.9		
rplN	Ribosomal protein L14	SP_0219		–5.1		
rpsN	Ribosomal protein S14	SP_0222		–2.6		
rplF	Ribosomal protein L6	SP_0225		–2.3		
rpsE	Ribosomal protein S5	SP_0227		–3.1		
rpmJ	Ribosomal protein L36	SP_0233		–2.7		
rplM	Ribosomal protein L13	SP_0294		–4.4		
rpmE	Ribosomal protein L31	SP_1299		–5.1		
tuf	Translation elongation factor Tu	SP_1489		–4.0		
rpmA	Ribosomal protein L27	SP_1107			2.1	
Transport						
adcB[a]	Zinc ABC transporter, permease protein	SP_2170	3.4			
aliA[a]	Oligopeptide ABC transporter, oligopeptide-binding protein AliA	SP_0366	3.0			
	Aquaporin	SP_1778	2.8			
psaC	Manganese ABC transporter, permease protein	SP_1649	2.4			
	ABC transporter, substrate-binding protein	SP_2197	–2.0			
msmK	Sugar ABC transporter, ATP-binding protein	SP_1580		2.5		
ptsL	Phospho-*enol*-pyruvate-protein phosphotransferase	SP_1176		–2.3		
	Non-heme iron-containing ferritin	SP_1572		–2.1		
	Iron-compound ABC transporter, iron-compound-binding protein	SP_1872			–4.7	
amiC	Oligopeptide ABC transporer, permease protein AmiC	SP_1890			2.5	
licC	PTS system, cellobiose-specific IIC component	SP_0474	4.2			
	PTS system, IIC component	SP_0062		3.8		
	PTS system, IIA component	SP_0064		2.8		
	PTS system, IID component	SP_2161				2.0

Table 3 (*continued*).

Function and gene name	Protein	TIGR4 acc. No.	Strain 82 1 h	Strain 82 3 h	Strain R6 1 h	Strain R6 3 h
Energy metabolism						
atpH	ATP synthase F1, Δ subunit	SP_1511	2.3		2.1	
sdhB	L-Serine dehydratase, iron-sulfur-dependent, β subunit	SP_0106	–2.8			
zwf	Glucose-6-phosphate 1-dehydrogenase	SP_1243	–2.5			
ccdA-2	Cytochrome *c*-type biogenesis protein CcdA	SP_0999	–2.1			
agaS	Sugar isomerase domain protein AgaS	SP_0065		4.2		
lacA	Galactose-6-phosphate isomerase, LacA subunit	SP_1193		2.3		
	Formate acetyltransferase	SP_0251		2.3		
	Glycosyl hydrolase, family 35	SP_0060		2.0		
gplK	Glycerol kinase	SP_2186			3.4	
Amino acid biosynthesis						
	Chorismate mutase	SP_1296	–3.1			
DNA metabolism						
xseB	Exodeoxyribonuclease VII, small subunit	SP_1206	–2.0			
holA	DNA polymerase III, Δ subunit	SP_0765	–2.0			
Fatty acid metabolism						
fabK	Enoyl-(acyl-carrier-protein) reductase	SP_0419	–2.7			
fabD	Malonyl CoA-acyl carrier protein transacylase	SP_0420	–2.0			
accC	Acetyl-CoA carboxylase, biotin carboxylase	SP_0425		–3.7		
Cell division						
divIVA	Cell division protein DivIVA	SP_1661			–2.8	
scpA	Segregation and condensation protein A	SP_1876			–2.0	
Transposon						
	IS630-Spn1, transposase Orf1	SP_0015			–2.2	
Purine and pyrimidine ribonucleotides biosynthesis						
purC[a]	Phosphoribosylaminoimidazole-succinocarboxamide synthase	SP_0044			–2.9	
	Phosphoribosylformylglycinamidine synthase	SP_0045			–3.4	
purF	Amidophosphoribosyltransferase	SP_0046			–6.5	
purM	Phosphoribosylformylglycinamide cyclo-ligase	SP_0047			–2.4	
purN	Phosphoribosylglycinamide formyltransferase	SP_0048			–4.0	
purH	Phosphoribosylaminoimidazolecarboxamide formyltransferase/ IMP cyclohydrolase	SP_0050			–2.4	
purK	Phosphoribosylaminoimidazole carboxylase, ATPase subunit	SP_0054			–3.2	
pyrDB	Dihydroorotate dehydrogenase B	SP_0964			–2.5	
guaA	GMP synthase	SP_1445			–2.3	
	Purine nucleoside phosphorylase, family 2	SP_0831			–2.2	
carA	Carbamoyl-phosphate synthase, small subunit	SP_1276			–3.3	
Proteins with unknown functions						
	Oxidoreductase, pyridine nucleotide-disulfide, class I	SP_1588	–2.9		–3.4	
	Glutamine amidotransferase, class I	SP_1089	–2.4			
NifR3 family	TIM-barrel protein	SP_2189	–2.4			
	Oxidoreductase	SP_0267			2.1	
	Acetyltransferase, GNAT family	SP_1943			–2.4	
	HIT family protein	SP_0521			–2.3	
	Oxidoreductase, Gfo/Idh/MocA family	SP_1482			–2.2	
	VanZ protein	SP_0049			–2.1	
Conserved hypothetical proteins						
	Conserved hypothetical protein	SP_0992	–2.7		–2.2	
	Conserved hypothetical protein	SP_0239	–2.2			
	Conserved hypothetical protein	SP_0276	–2.2			
	Conserved hypothetical protein	SP_0822			–2.2	

Table 3 (*concluded*).

Function and gene name	Protein	TIGR4 acc. No.	Strain 82 1 h	Strain 82 3 h	Strain R6 1 h	Strain R6 3 h
Hypothetical proteins						
	Hypothetical protein	SP_0157	3.2		2.1	
	Hypothetical protein	SP_1465	–3.2			
	Hypothetical protein	SP_0311	–2.1			
	Hypothetical protein	SP_0703		2.4		
	Hypothetical protein	SP_1924		–3.8		
	Hypothetical protein	SP_1925		–6.1		
	Hypothetical protein	SP_1926		–3.5		
	Hypothetical protein	SP_0781			–2.6	
	Hypothetical protein	SP_0830			–2.6	
	Hypothetical protein	SP_0055			–2.0	

[a]Genes associated with pathogenesis but belong to other functional categories.

all the analyzed genes changed or unchanged accordingly but at a greater average fold change in qRT-PCR analysis (Fig. 1).

Mutagenesis works in *S. pneumoniae*

We sought to further characterize the functions of the microarray identified genes by knockout mutagenesis in the same analyzed strains. Unfortunately, we were unsuccessful in transforming linear PCR fragments into strain 82 and a few other serotype 3 clinical isolates using different transformation methods (Bricker and Camilli 1999; Johnston et al. 2006) and CSP peptides (data not shown). A low level of competence appeared to be a major obstacle for the genetic studies of serotype 3 pneumococcal strains (Hsieh et al. 2006). We therefore constructed knockout mutants in strain R6 (spr), where the SP_ homolog genes were disrupted (Fig. 2). To be consistent with microarray data, all the mutants were designated as TIGR4 genome accession identity (SP_). Considering the importance of the regulatory and transport genes and the location in the genomes, we targeted 3 differentially regulated genetic loci (*i*) the down-regulated SP_0416 in strain 82 at both 1 and 3 h, which encodes a MarR family transcriptional regulator; (*ii*) the up-regulated SP_2170 (*adcB*) in strain 82 at 1 h, encoding a zinc ABC transporter; and (*iii*) a hypothetical gene (SP_0157), which was up-regulated in both strains 82 and R6 at 1 h (Table 3). As we were interested in the identification of potential functional regions in this work, partial flanking region sequences were also disrupted in some constructed mutants, such as the 3′-end of SP_0415 (enoyl-CoA hydratase/isomerase family protein) in ΔSP_0416 (Fig. 2A) and the 5′-end of the SP_0158 (unknown function) in ΔSP_0157 (Fig. 2C). We did not observe apparent changes in growth rates for the constructed mutants when grown in THY media (data not shown).

To examine if there were any polar effects in the constructed mutants because of the insertion of the Em cassette, we further analyzed mRNA expression levels of the genes located downstream of the disrupted genes by qRT-PCR. Compared with the parent strain R6, we found that the expression of SP_2169 increased 23.7-fold in the ΔSP_2170 mutant, and SP_0158 and SP_0159 were increased 2.8-fold and 4.4-fold, respectively, in the ΔSP_0157 mutant. It indicates that the expression of downstream genes was changed in both of the tested mutant strains. An enhanced expression of these downstream genes is possibly due to the promoter of the Em cassette, which was inserted in the same orientation.

Interestingly, when cDNA samples of strain R6 and the ΔSP_0157 mutant were amplified with primer pairs of SP_0158 (sense) and SP_0159 (anti-sense) (Table 2), a PCR product could be identified in the SP_0158 and SP_0159 intergenic region (data not shown), showing that both SP_0158 and SP_0159 may express in one transcript despite their opposite orientation in the genome sequence (Fig. 2C). This finding also explained the phenomenon for an enhanced expression of SP_0159 in the ΔSP_0157 mutant.

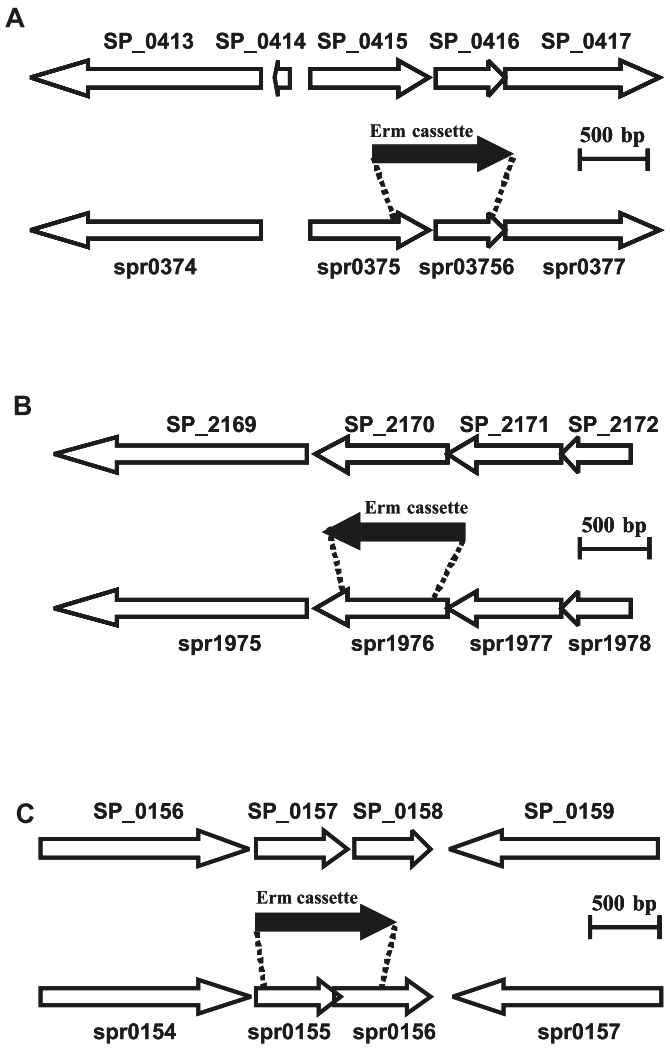
Adherence activities

To investigate functions of the selected genes, we further examined activities of the mutant strains in their adherence to A549 cells. At a multiplicity of infection of 10:1, the ΔSP_2170 mutant strain adhered to A549 cells at a much lower level than the parent strain R6 at both 1 and 3 h incubation time periods. Such a difference was also observed in the ΔSP_0157 mutant strain when incubated with A549 cells for 3 h. However, no difference was observed for the ΔSP_0416 mutant (Fig. 3). A similar phenomenon was also observed for these strains when the experiment was performed at a higher multiplicity of infection (data not shown).

Discussion

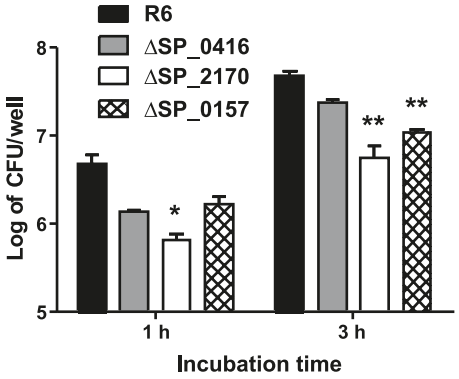
To investigate pathogen–host interactions and microbial pathogenesis, transcriptional microarray analysis has been applied in a few pathogen studies, such as the group A streptococcus (GAS) (Musser and DeLeo 2005). In *S. pneumoniae*, gene transcriptional responses to host epithelial cells, blood, and cerebrospinal fluid (CSF) have been investigated (Orihuela et al. 2004). In host epithelial cells, Orihuela et al. (2004) examined gene expression changes of a serotype 4-derived unencapsulated strain upon exposure to human nasopharyngeal epithelial cells (Detroit 562) for 3 h. Considering the strain-specific gene regulation features in *S. pneumoniae* (Hendriksen et al. 2007), we investigated

Fig. 2. Diagrammatic representation of the mutant strains constructed in *Streptococcus pneumoniae* R6 (spr). The correlated genetic regions in *S. pneumoniae* TIGR4 (SP_) are mapped on the top according to the genome sequences of strains R6 and TIGR4 (TIGR). (A) Genes disrupted in the SP_0416 genetic locus; (B) genes disrupted in the SP_2170 genetic locus; and (C) genes disrupted in the SP_0157 genetic locus. The genetic regions (open arrows), replaced by the Erm resistance cassette (solid arrow), are indicated with dashed lines.



gene expression changes of an encapsulated serotype 3 strain and an unencapsulated avirulent strain upon exposure to human lung epithelial cells (A549) for 1 and 3 h, respectively. In this study, we observed divergent gene expression changes in 2 strains, and many genes associated with pathogenesis, cell envelope, transcription, translation, transport, and metabolism were only changed in the pathogenic strain 82 (Table 3). Although such divergent transcriptional responses could be explained by the difference in their serotypes (Bruckner et al. 2004; Silva et al. 2006) and by the mutations that occurred in the avirulent strain R6 (Lanie et al. 2007; Williams et al. 2007), there was still a big difference between pathogenic and avirulent strains in their gene responses to host epithelial cells. Such a difference was also

Fig. 3. Analysis of bacterial adherence to A549 cells for the mutant strains derived from *Streptococcus pneumoniae* R6. Adherent bacterial colony-forming units (CFU) to host cells (in logarithmic scale) is indicated on the *y*-axis. The standard deviation is marked with error bars. Statistical significance of adherence between parent strain R6 and isogenic mutant strains at each time point is marked with asterisks obtained from the Prism *t*-test analysis. *, statistically significant at $P < 0.05$; **, statistically significant at $P < 0.01$ or 0.005.



observed in a separate microarray analysis in which both R6 and its pathogenic progenitor strain D39 were investigated (X.-M. Song, W. Connor, K. Hokamp, unpublished data).

As we expected, some gene expression changes in the pathogenic strain 82 were also observed in the previously studied serotype 4-derived pneumococcal strain, such as the *lic* operon genes (SP_1268, SP_1269, SP_1270, SP_1271), *ply* (SP_1923), *aliA* (SP_0366), and *psaC* (SP_1649) (Orihuela et al. 2004). However, not as many genes changed significantly in our microarray analysis. It is probably due to the differences in the studied strains, host cells, and the microarray analysis. Sequence variation between the tested samples (serotype 3 and serotype 2-derived) and microarray probes (designed from serotype 4) might be a major reason for the negative or weak signals for some sequence-divergent genes in our studies.

Streptococcus pneumoniae serotype 3 strains are becoming a concern in the clinic, as they are not protected by the currently used 7-valent conjugated pneumococcal polysaccharide vaccine (PCV-7) (Kronenberg et al. 2006). Although the virulence of a serotype 3 pneumococcal strain has been studied with signature-tagged mutagenesis in a murine respiratory tract infection model (Lau et al. 2001), the pathogenesis of serotype 3 strains is still largely unknown. Microarray analysis, supplemented with the possible availability of *S. pneumoniae* serotype 3 genome sequences (http://www.sanger.ac.uk/Projects/S_pneumoniae/), will enable us to gain more insights into serotype 3 pneumococcal pathogenesis.

Among the pathogenesis genes, we found *ply* (SP_1923) expression was reduced at 3 h in strain 82 in both microarray and qRT-PCR assays (Fig. 1A). The *ply* gene encodes pneumolysin, which causes host cell death via cytotoxicity and plays a major role in pneumococcal invasive infections (Hirst et al. 2004). Down-regulation of *ply* suggests that pneumolysin might not be required during an initial stage of interaction with host epithelial cells. Interestingly, in strain

82, expression of 3 hypothetical genes (SP_1924, SP_1925, SP_1926) located upstream of *ply* was also down-regulated under the same conditions (Table 3). Since there is only a 12 bp intergenic region between *ply* and the immediate upstream SP_1924 (TIGR), it is likely they are co-expressed on the same operon. Another regulated virulence gene was *bgaA* (SP_0648); its expression was up-regulated in strain 82 at 3 h in both the microarray and qRT-PCR assays (Fig. 1A). Together with NanA (neuraminidase) and StrH (β-*N*-acetylglucosaminidase), BgaA (β-galactosidase) contributes to the pneumococcal adherence to human epithelial cells (King et al. 2006). The *lic* operon genes, including *licB* (SP_1268), *pck* (SP_1269), SP_1270, and SP_1271, were also up-regulated in strain 82 (Table 3; Fig. 1A). The *lic* operon genes are responsible for the incorporation of phosphocholine (*P*Cho) residues into lipoteichoic (LTA) and teichoic acid (TA) on the membrane, while the expression of choline-binding family proteins on the surface is dependent on their binding to the incorporated *P*Cho. Inactivation of the *lic* genes results in a dramatic reduction in pneumococcal virulence (Kharat and Tomasz 2003). Therefore, enhancement of *P*Cho incorporation into the pneumococcal membrane appears to be needed at an early stage of infection for the expression of choline-binding proteins, one of the major components contributing to the colonization of host epithelial cells.

One striking observation in strain R6 was that expression of 11 genes associated with ribonucleotide biosynthesis was down-regulated after 1 h incubation (Table 3). The expression changes of *purF*, *purK*, and *carA* were verified by qRT-PCR analysis (Fig. 1B). In *Bacillus subtilis*, expression of the *pur* gene cluster is regulated by a negative transcriptional regulator *purR* (Sinha et al. 2003). However, expression of the *purR* homolog gene in strain R6 was unchanged in our microarray and qRT-PCR analyses (Fig. 1B). In other studies, the expression of pneumococcal ribonucleotide biosynthesis genes is up-regulated when incubated in animal blood (Orihuela et al. 2004) or when treated with sublethal concentrations of antibiotics (Ng et al. 2003). It suggests that the ribonucleotide biosynthesis genes play a role in pathogen–host interactions. However, since those genes were only changed in the avirulent strain in this study (Table 3), it casts some doubt on the value of those genes in pathogenesis during interaction with host epithelial cells.

In this study, we inactivated several genetic loci by allelic-homologous replacement and characterized the roles of the constructed mutants in adherence to A549 cells. The first mutated genetic locus includes a MarR family transcriptional regulatory gene SP_0416, which was down-regulated in strain 82 (Table 3; Fig. 2A). The MarR (multiple antibiotic resistance regulator) family of prokaryotic transcriptional regulators includes proteins critical for control of virulence factor production, bacterial response to antibiotic and oxidative stresses, and catabolism of environmental aromatic compounds (Wilkinson and Grove 2006). In *S. pneumoniae*, SP_0416 is located within a type II fatty acid biosynthesis gene cluster, thus, playing a role in regulating *fab* fatty acid biosynthesis genes in strain R6 (Lu and Rock 2006). In our analysis, disruption of SP_0416 in strain R6 did not cause any changes in adherence to A549 cells (Fig. 3). However, when SP_0416 was disrupted in encapsulated pathogenic strains, it appeared to be detrimental to the strains (data not shown), indicating different roles of SP_0416 in pneumococcal strains.

The second disrupted genetic locus contains *adcB* (SP_2170), which was induced in strain 82 (Table 3; Fig. 2B). An enhanced expression of *adcB* suggests that bacteria might face a zinc-restricted environment when incubated with host cells as opposed to the zinc content of rich media. In *S. pneumoniae*, the *adc* operon-encoded zinc ABC transport system regulates growth and competence (Dintilhac and Claverys 1997). Together with the *psa* operon-encoded manganese ABC transport system, zinc and manganese transport systems play an essential role in pneumococcal survival (Dintilhac et al. 1997). In this study, the manganese ABC transporter gene, encoded by *psaC* (SP_1649), was also induced in strain 82 (Table 3). The *psa* operon has been well characterized (McAllister et al. 2004), whereas the roles of *adc* operon in adherence to host cells are still unclear. In our adherence analysis, the ΔSP_2170 mutant exhibited a much lower level than the parent strain R6 at both 1 and 3 h (Fig. 3), indicating that SP_2170 plays a role in adherence to host epithelial cells. As transport of zinc requires all the components encoded by the *adcRCBA* operon genes, the reduced adherence activity is probably mediated by the disrupted zinc transport system. An impaired zinc transport system could further reduce the expression of some laminin adhesion (Lmb) and pneumococcal histidine triad (PHT) proteins that are likely involved in the adhesion and invasion process (Panina et al. 2003).

The third characterized genetic locus contains a hypothetical gene (SP_0157), which was up-regulated in both 82 and R6 strains (Table 3). In strain R6, the promoter region of the SP_0158 homolog gene is located inside of the SP_0157 homolog coding region, indicating that they are very likely expressed as one transcript. We therefore knocked out both genes in the constructed ΔSP_0157 mutant (Fig. 2C). Further analysis of this mutant strain revealed a much lower level of adhesion activity than the parent strain R6 at a 3 h incubation time period (Fig. 3). Downstream of SP_0158, there are 3 hypothetical genes (SP_0159, SP_0160, SP_0161) oriented in the opposite direction (TIGR). However, our data indicated that SP_0157 and SP_0158 transcripts may interact with these genes in gene expression. At least one transcript could be detected in the SP_0158 and SP_0159 intergenic region in our RT-PCR analysis, and no apparent transcription terminator structures could be identified in this region when mRNA sequence was folded (data not shown). A coordinated expression of these hypothetical genes might be required for the observed adherence activity.

In this study, to investigate genes associated with pathogen–host interactions, we examined gene expression changes of pneumococci during interaction with human lung epithelial cells. We found many categories of genes were differentially regulated in the pathogenic strain but few of them changed in the avirulent strain. It suggests that pathogenic and avirulent strains exhibited divergent gene responses to host cells. Some of those gene expression changes might be associated with pathogenesis. To support this hypothesis, we analyzed 3 differentially regulated genetic loci by insertional mutagenesis and found 2 were

required for complete adherence to human lung epithelial cells in strain R6. The specific roles of each individual gene have yet to be further investigated.

Acknowledgements

We thank Ms. Stacie Skiftun and Mr. Wenkai Fu for their excellent technical contributions and Dr. Wolfgang Koster for his valuable discussions. This manuscript was critically reviewed by Dr. Robert Brownlie. We also thank Dr. Donald E. Low of Mount Sinai hospital in Toronto for providing pneumococcal strains, Dr. Donald A. Morrison of University Illinois at Chicago for providing Erm-resistance cassette, and Dr. Ramkumar V. Iyer of Tufts University for providing transformation protocols. This work was supported by the Saskatchewan Health Research Foundation (SHRF) and the University of Saskatchewan Delfari Bridging Fund to Xin-Ming Song. Andrew A. Potter is supported by an NSERC Senior Industrial Research Chair. Published with permission of the Director of VIDO as journal series No. 450.

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